REPORT ON FORMETANATE HCL: DOSE-RESPONSE AND TIME-COURSE IN PND17 RATS

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July 15, 2009

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Introduction

The *N*-methyl carbamate pesticides inhibit acetylcholinesterase, producing cholinergic overstimulation that can alter activity levels, cause autonomic and neuromuscular dysfunction, and at high doses, result in coma and death. While carbamates have been widely studied for decades, almost all such studies have used only adult laboratory animals. There are very few studies in the literature concerning differential sensitivity of the young to carbamates as a class, and none that we could locate for formetanate specifically.

We have evaluated a series of carbamates using behavioral and biochemical endpoints to learn more about potential age-related differences. The time-course of brain and RBC cholinesterase (ChE) inhibition was established followed by dose-response evaluation at the approximate time of peak effect. Behavior was monitored in motor activity chambers, and ChE inhibition was measured using a radiometric assay that minimized reversal. This latter factor is a key issue in conducting *ex vivo* assays of ChE inhibition in carbamate-treated tissues.

This report provides the results of the time-course and dose-response study of formetanate HCl in 17-day old Long-Evans rats. These data, along with those of the other carbamates, are summarized in manuscripts that are in preparation for journal submission.

Methods

Chemicals

We obtained analytical grade (99% pure) formetanate HCl (CAS #23422-53-9; lot# 341-144B) from Chem-Service Inc (West Chester, PA). Deionized water was used as the vehicle. The high concentration was prepared, and serial dilutions were performed to make the lower concentrations. Fresh dosing solutions were prepared each day.

For the ChE assay, [^{3}H]acetylcholine iodide (76 mCi/mmol; Perkin Elmer Life Sciences, Boston, MA) and other reagents (Sigma-Aldrich, St. Louis, MO) were obtained at reagent-grade purity.

Animals

Timed-pregnant Long-Evans rats (Charles River Laboratories, Raleigh, NC) were individually housed on hardwood chip bedding (Beta-Chip®) with a cotton pad (Nestlet®) in each cage to serve as nesting material. Food (Purina Formulab Diet #5008) and water (filtered tap) were freely available. They were housed in the AAALAC International-accredited animal facility with regulated temperature (72±2°C) and humidity (50%±20%). These set points were met with a few exceptions: one day with a high temperature of 75°C, and two days with low humidity (22%, 28%).

Rats were allowed to deliver naturally; day of birth is considered postnatal day (PND) 0. All births that take place within a 24-hr period are considered as the same day of birth. On PND3 (time-course) or PND2 (dose-response), all pups were grouped by sex and redistributed to the dams, assuring that littermates are spread across litters. All litters

were culled to 8 pups, with 6 males in each. Only males were used in these studies. Rat pups were identified by stripes marked on their tails at dosing, using non-toxic markers.

In-Life Testing

On the day of dosing, rats were weighed to calculate the injection volumes. The doses were given at 2 ml/kg, administered orally using 22G stainless steel gavage needles (Popper and Sons, Lake Success, NY). Five male pups in each litter were dosed in a split-litter design, *i.e.*, no more than one pup within a litter received the same dose. Dosing was spaced so that sacrifice and tissue collection can take place at the same approximate time after dosing for all pups within the treatment group. After dosing, pups were placed back in their home cage until testing. To the extent possible, treatments were counterbalanced across the days of testing (note, the treatment groups for the longer time points were dosed earlier in the day). The time-course and dose-response studies were separated by 10 months.

For the time-course, rats (n=6/dose at each time) were dosed with either vehicle (deionized water) or formetanate 3 mg/kg. Nominal time points for the time-course study were 15, 45, 90, 180, or 1440 min (24 hr); in practice, precise times were 15-20, 45-55, 90-95, 180-190, and 1440-1450 min after dosing. Control rats were included only at 45, 180, and 1440 min. For the dose-response study, rats (n=10/dose) were dosed with 0, 0.1, 0.3, 0.75, or 1.5 mg/kg formetanate, and euthanized at 40-45 min after dosing.

The motor activity study was conducted only in the dose-response study. Fifteen min after dosing, rats were placed in the activity chamber shaped like a figure-eight (Reiter 1983) and housed in individual ventilated cabinets with white noise. Photobeams spaced around the chamber detected movement as counts that were tabulated in 5-min intervals for a total of 20 min.

Tissue Collection

At the appropriate time in the time-course study, or immediately after the motor activity assessment in the dose-response study, rats were decapitated quickly under light CO_2-induced anesthesia. Trunk blood was collected in heparinized tubes. The whole brain was removed from the skull and split sagitally. The two halves were put in separate microcentrifuge tubes and placed in dry ice for quick freezing.

After no more than 10 minutes, the tubes with blood were placed in the table top centrifuge and spun at 1000g for 10 minutes. A minimum of 200 µl plasma was placed into another microcentrifuge tube. After changing tips, 200 µl of the packed RBC was removed and placed in another tube with 400 µl 0.1 M $NaPO_4$, pH 8.0/1.0% Triton X-100 buffer. Tubes were vortexed briefly. This allowed a 1:2 dilution (1 part plus 2 parts) of the RBC. The tubes with plasma and RBC were then placed in dry ice. At the end of the day's tissue collection, all samples were placed in plastic bags, labeled with the study, date, and age, and placed in a freezer at -80°C.

Cholinesterase Assay

A radiometric assay (Johnson and Russell 1975) was used to determine ChE activity in RBC and one of the half-brains. On the day of assay, the tubes were placed in ice for slow thawing. Each half-brain was scooped from the tube, weighed, and placed into a larger pre-numbered tube in ice for homogenizing. An appropriate amount of 0.1

M $NaPO_4$, pH 8.0/1.0% Triton X-100 buffer was added to give a 1:2 dilution (for example, a 1.4 g brain had 2.8 ml buffer added to it). This was then homogenized using the Polytron homogenizer (Kinematica Model PT3100, Littau, Switzerland) set at 10,000 rpm for 10-30 sec. The Polytron generator was rinsed in clean water and excess water blotted off before the next sample was homogenized. After homogenizing, each tube with the brain homogenate was quickly returned to the ice. Unused brain homogenates and RBCs were returned for storage at -80°C.

The ChE assay was run using 20 µl of the sample and 80 µl substrate containing 0.1 µCi [^{3}H]-acetylcholine iodide in a final concentration of 1.2 mM. RBC samples were incubated for 2 minutes, brain samples for 30 sec. Reactions took place in a water bath at 26ºC. This small volume, low temperature, and fast reaction of the assay minimize tissue dilution and decarbamylation. The enzyme activity was ended using the stop solution, scintillant was added and the tubes shaken briefly, and the amount of ^{3}H-acetate in the upper phase was counted using a liquid scintillation counter (model LS6500, Fullerton, CA). Counting efficiency, as determined by an external quench standard, was approximately 62%. All samples were run in duplicate; any duplicates >20% apart were excluded or re-run in the assay. Only one sample exceeded this criterion and was excluded.

Quality Assurances

All experimentation was conducted under approved laboratory and animal research protocols and standard operating procedures, according to the NHEERL Quality Assurance Management Plan. All systems checks and data objectives were met in both studies. These specific data were audited and approved by NHEERL Quality Assurance managers; see Appendix 2 for the audit report.

Statistical Analyses

Data for formetanate-treated rats at each time point on the day of dosing were subjected to ANOVA, followed by Tukey's test to determine differences across times. The 24-hr treated and control groups were compared separately. Dose-response data (ChE and activity counts) were analyzed using ANOVA followed by Dunnett's t-test to determine dose groups that were different from control. Probability values <0.05 were considered statistically significant; RBC and brain data were analyzed separately.

Results

There were no unanticipated deaths or cases of severe toxicity. The raw data are presented in Appendix 1.

Table 1 presents ChE activity for each control group; these values were similar across time in the time-course, and between the time-course and dose-response studies. For purposes of presentation, ChE activity data are graphed as percent of the relevant control data (for the time-course, control groups on the day of dosing combined, 24-hr control separate); statistical analyses were conducted on the untransformed data.

Table 1. Control ChE activity as umoles ACh hydrolyzed/minute/g brain or ml RBC, mean ± SEM.

Time point	Brain	RBC
Time-course 45 min	5.222 ± 0.081	0.700 ± 0.057
Time-course 180 min	5.524 ± 0.082	0.767 ± 0.057
Time-course 1440 min	5.422 ± 0.070	0.783 ± 0.054
Dose-response 40 min	5.788 ± 0.142	0.878 ± 0.045

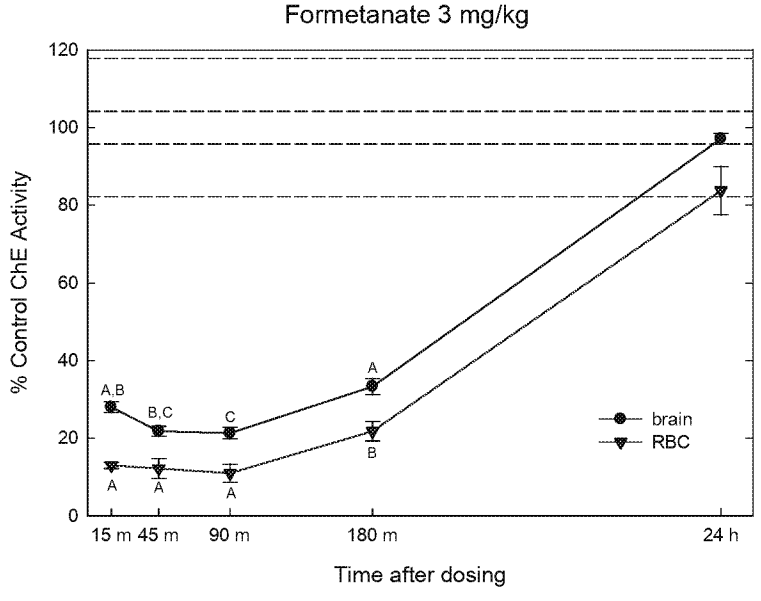
Time-Course

Both brain and RBC ChE showed considerable inhibition of ChE activity (approximately 80% and 90% inhibition in brain and RBC, respectively) on the day of dosing, with recovery at 24 hr. The overall time effect on the day of dosing was significant for both tissues (brain $F_{(3,20)}$=12.84, $p<0.0001$; RBC $F_{(3,20)}$=5.18, $p=0.0082$). These data are shown in Figure 1.

For brain ChE, the first two time points (15, 45 min) were not significantly different, while 15 min was significantly greater than the 90 min time point. Both the 45 and 90 min data were significantly less than the 180 min. Thus, the time of peak effect could be considered later >15 min and ≤90 min. The treated group was not significantly different from control at 24 hr. Our choice of 40-45 min for the dose-response was based on these data as well as those for the other carbamates being tested, and our need to have a common test time for all carbamates.

The RBC ChE data showed that all three early time points (15, 45, and 90 min) were significantly lower than the 180 min data. As with the brain data, the treated group was not significantly different from control at 24 hr.

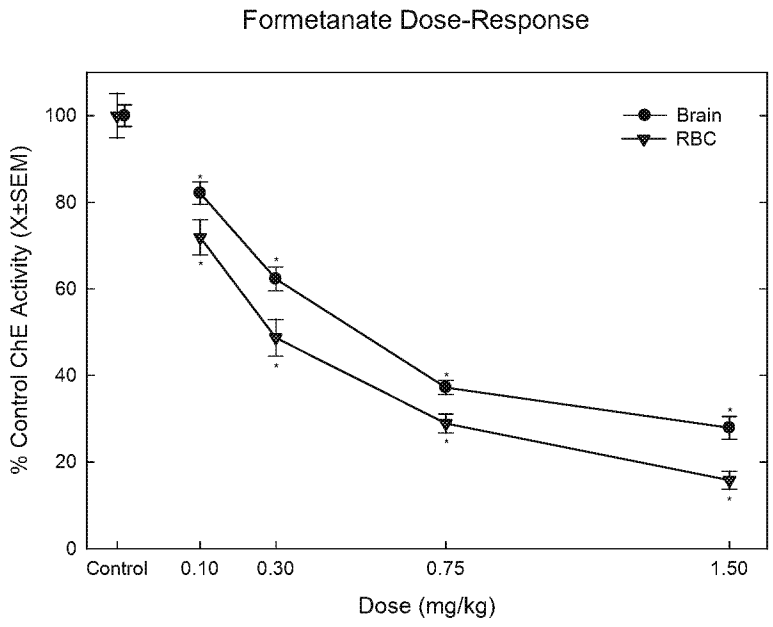
Figure 1. Time-course of ChE activity as % control (see above), mean ± SEM. Dashed line indicates ±1SD around the control data. Means with the same letter are not significantly different (for brain and RBC separately).



Dose-Response

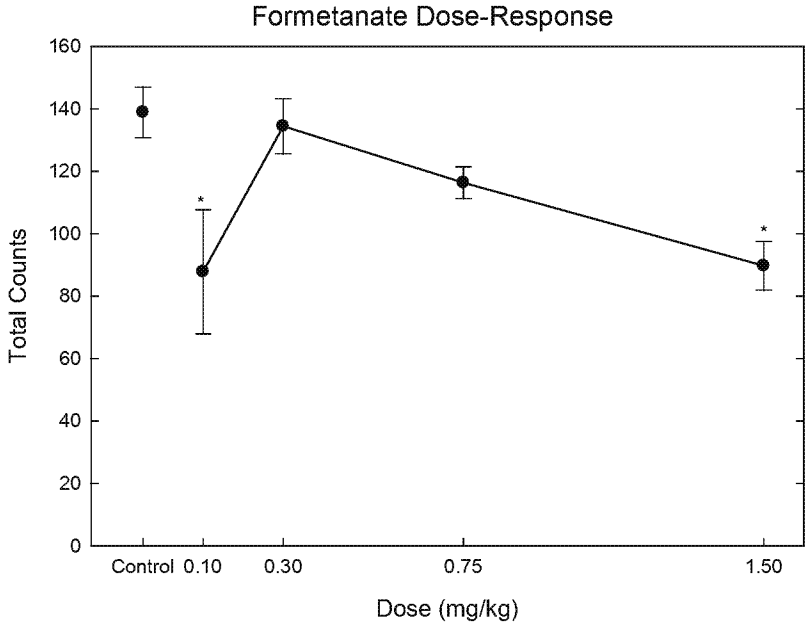
As seen in Figure 2, brain and RBC ChE were decreased (brain $F_{(4,45)}$=154.12, $p<0.0001$; RBC $F_{(4,45)}$=81.74, $p<0.0001$) in a treatment-related manner, and all doses were significantly different from control. Thus, a NOAEL could not be determined. The lowest dose tested (0.1 mg/kg) produced 17.9% and 28.1% inhibition in brain and RBC, respectively.

Figure 2. Dose-response of ChE activity as % control, mean ± SEM. * indicates dose groups significantly different from control.



In contrast to the ChE data, motor activity did not show a monotonic dose-response. While the overall ANOVA was significant ($F_{(4,45)}$=4.62, p=0.0033), only the lowest and highest doses decreased activity. As seen in Figure 3, the data in the lowest dose group were highly variable.

Figure 3. Dose-response of motor activity (total counts over the session) as % control, mean ± SEM. * indicates dose groups significantly different from control.



Discussion

The time-course and degree of inhibition in the pups dosed with formetanate 3 mg/kg was very similar to that seen at 10 mg/kg in adults (Padilla *et al.* 2007). At all time points, RBC inhibition was greater than brain in the young rats, whereas this was not the case with adults (Padilla *et al.* 2007). While the exact same times were not tested in the previous paper and the current study, it was apparent in both ages that onset of ChE inhibition occurred quickly (by 30 min) and recovered within one to two hours.

Both brain and RBC ChE were significantly different from control at the lowest dose, 0.1 mg/kg. In contrast, that dose did not alter ChE in adult rats (McDaniel *et al.* 2007). As in the time-course study, RBC was inhibited to a greater degree than brain at all doses, in contrast to the pattern seen in adult rats (McDaniel *et al.* 2007).

Motor activity was not a sensitive or consistent indicator of formetanate toxicity in this study. While the lowest dose was significantly lower than controls, that finding is questionable considering the wide range of counts that were obtained in that dose group. The highest dose group, which was significantly different from control, produced only a moderate decrease in activity. These data stand in marked contrast to the close correlations between ChE inhibition and motor activity decreases reported in adult rats (McDaniel *et al.* 2007). However, an inverted U-shape dose-response was also reported in PND17 rats for another carbamate, aldicarb (Moser 1999), and atypical dose-response curves have been recorded by other carbamates in other PND17 studies in this laboratory (manuscript in preparation). While the explanation for this age-related difference is

unclear, it may be that motor activity is not a sensitive indicator of behavioral dysfunction in preweanling rats, as opposed to adults.

Our literature search did not reveal any studies documenting the effects of formetanate in preweanling rats, thus these data provide an important contribution to our growing understanding of carbamate effects in the young.

References

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McDaniel, K. L., Padilla, S., Marshall, R. S., Phillips, P. M., Podhorniak, L., Qian, Y., and Moser, V. C. (2007). Comparison of acute neurobehavioral and cholinesterase inhibitory effects of N-methylcarbamates in rat. *Toxicol. Sci.* **98**(2), 552-560.

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Appendix 1. Raw data.

Time-course

Pup#	dose (mg/kg)	time (min)	brain (umole/min/g)	rbc (umole/min/ml)
613001	3	15	1.26	0.087
613101	3	15	1.491	0.111
613201	3	15	1.473	0.09
613301	3	15	1.645	0.077
613501	3	15	1.39	0.116
613801	3	15	1.783	0.091
613004	0	45	5.159	0.523
613204	0	45	4.956	0.85
613304	0	45	5.204	0.571
613604	0	45	5.57	0.859
613702	0	45	5.234	0.69
613806	0	45	5.206	0.709
613003	3	45	1.402	0.174
613203	3	45	1.076	0.05
613303	3	45	1.194	0.095
613603	3	45	1.259	0.093
613701	3	45	0.895	0.055
613805	3	45	1.197	0.07
613002	3	90	0.953	0.068
613102	3	90	1.475	0.161
613202	3	90	1.042	0.041
613302	3	90	1.249	0.088
613502	3	90	1.021	0.066
613802	3	90	1.135	0.061
613104	0	180	5.628	0.785
613206	0	180	5.53	0.763
613504	0	180	5.635	0.59
613602	0	180	5.134	0.687
613704	0	180	5.525	1.01
613804	0	180	5.692	0.768
613103	3	180	2.103	0.173
613205	3	180	1.531	0.119
613503	3	180	1.944	0.221
613601	3	180	1.687	0.165
613703	3	180	1.448	0.097
613803	3	180	2.034	0.186
613006	0	1440	5.668	0.839
613106	0	1440	5.831	0.699
613306	0	1440	5.745	0.768
613506	0	1440	5.565	.
613606	0	1440	5.624	0.958
613706	0	1440	5.045	0.65
613005	3	1440	5.216	0.583
613105	3	1440	5.402	0.641

613305	3	1440	5.322	0.469
613505	3	1440	5.726	0.693
613605	3	1440	5.448	0.779
613705	3	1440	5.417	0.77

Dose-response

rat#	dose (mg/kg)	brain (umole/min/g)	rbc (umole/min/ml)	activity counts
638601	0	6.224	1.029	126
638802	0	5.823	0.892	142
638903	0	6.403	0.984	160
639004	0	5.197	0.638	156
639105	0	5.601	0.892	148
639201	0	5.39	0.889	137
639302	0	6.282	0.926	109
639403	0	5.138	0.943	183
639504	0	5.993	0.976	93
639705	0	5.828	0.608	135
638602	0.1	4.64	0.461	151
638803	0.1	5.573	0.723	113
638904	0.1	4.019	0.482	10
639005	0.1	5.269	0.84	87
639101	0.1	4.811	0.669	204
639202	0.1	5.157	0.628	120
639303	0.1	4.708	0.575	39
639404	0.1	4.491	0.649	32
639505	0.1	4.567	0.581	19
639701	0.1	4.284	0.703	103
638603	0.3	3.966	0.467	125
638804	0.3	2.621	0.327	149
638905	0.3	4.071	0.543	148
639001	0.3	3.697	0.44	179
639102	0.3	3.749	0.535	138
639203	0.3	3.05	0.268	76
639304	0.3	3.468	0.405	143
639405	0.3	4.083	0.32	154
639501	0.3	4.065	0.627	116
639702	0.3	3.319	0.341	116
638604	0.75	2.003	0.208	142
638805	0.75	2.136	0.19	123
638901	0.75	1.98	0.178	105
639002	0.75	2.081	0.237	124
639103	0.75	2.191	0.314	109
639204	0.75	2.658	0.362	134
639305	0.75	2.53	0.309	88
639401	0.75	2.385	0.276	125
639502	0.75	1.924	0.224	109
639703	0.75	1.664	0.24	104
638605	1.5	1.408	0.107	95

638801	1.5	1.442	0.134	114
638902	1.5	1.04	0.081	67
639003	1.5	1.154	0.074	75
639104	1.5	1.958	0.119	90
639205	1.5	2.627	0.196	129
639301	1.5	1.619	0.194	117
639402	1.5	1.471	0.112	88
639503	1.5	2.081	0.26	70
639704	1.5	1.365	0.113	52

Appendix 2.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
National Health and Environmental Effects Research Laboratory
Research Triangle Park, NC 27711

OFFICE OF
RESEARCH AND DEVELOPMENT

June 26, 2009

MEMORANDUM

SUBJECT: Quality Assurance Inspection Statement to Accompany Formetanate Research Study Report

FROM: Carol T. Mitchell, *CTM June 26, 2009*
Quality Assurance and Records Manager, Toxicity Assessment Division (MD 71)

Brenda T. Culpepper, *btc June 26, 2009*
NHEERL Director of Quality Assurance (MD B343-01)

TO: Virginia C. Moser, Ph.D., Toxicity Assessment Division (MD B105-04)

The primary objective of this Quality Assurance Data Review of the Formetanate Study was to provide assistance to you; to help ensure that the study quality assurance (QA) and quality control (QC) procedures were appropriate for the anticipated end use of the data and that study documentation was adequate to ensure the defensibility of the study results.

The Formetanate study was conducted under Amendment #1, IRP NHEERL-RTP/NTD/NBTB/VCM/2003-02-001, "Age-Related Neurotoxicity of Pesticides: Suseptible Populations".

The original IRP described behavioral and biochemical studies in young rats exposed to organophosphate pesticides. At the time of writing the IRP, Dr. Moser considered similar studies using *N*-methyl carbamate pesticides, and included the following sentence in the original: "Additional pesticides, *e.g.*, carbamates, may be examined in the future." Several years into the research, the carbamates were added and the Laboratory Animal LAPR was amended to include them. Amendment #1 describes the addition of the following carbamates to the list of chemicals that were examined under this IRP: formetanate, methomyl, oxamyl, methiocarb, and propoxur. These chemicals are purchased from Chem Serv, Inc. at the highest purity available. Most of the chemicals are not highly toxic and therefore do not require Safety Protocols; however, there is an approved protocol for the handling of the few that are highly toxic.

The study was well planned, organized and executed. All study participants are to be commended for their effort in executing the study with a high degree of expertise and integrity ensuring the overall quality of the study. The research team consisting of Dr. Virginia C. Moser, PI; and Pamela Phillips and Katherine McDaniel, Biologists, have worked closely together for more than 20 years and are both experienced and highly qualified to perform the work.

The laboratories and equipment are well maintained and are adequate to produce the results of a quality sufficient to meet the objectives of the study. The Formetanate study was conducted as a QA Category IV, basic research and development study, and was not audited by the division QA Manager during the life of the study. However, other carbamate studies following the same protocols received in-life surveillances and a Technical Systems Review (QA audit) from NHEERL Quality Assurance Staff, because the studies were requested by the Office of Pesticide Programs and considered to be QA Category II studies.

The QA review team consisted of Brenda T. Culpepper, NHEERL Director of Quality Assurance and Assistant Director of Records Management, and Carol T. Mitchell, QA and Records Manager of the NHEERL Toxicity Assessment Division. They began this QA data audit by checking every individual raw data point from the Formetanate study. The raw data points were compared to spreadsheets coded with animal weights, dose groups, and brain, blood, and motor activity data and were traced to the Radiometric acetylcholinesterase assay results that measured levels of ChE in the blood and brain tissues. When no discrepancies were found, the QA review team finished the data review by randomly selecting from each dose group and tissue type. We were able to track back from each final acetylcholinesterase activity value to individual animals within each Formetanate dosage or control group, finding no discrepancies. Below the activity, dates, and participants are listed.

DATA AUDIT

• Tuesday, 16 June 2009 (a.m.)	Carol Mitchell and Brenda Culpepper
• Tuesday, 16 June 2009 (p.m.)	Carol Mitchell
• Tuesday, 16 June 2009 (p.m.)	(Carol Mitchell with Pam Phillips —1 hour as Technical Representative to answer questions)
• Thursday, 25 June 2009 (a.m.)	Carol Mitchell and Brenda Culpepper
• Thursday, 25 June 2009 (a.m.)	Carol Mitchell, Brenda Culpepper, and Virginia Moser (1 hour as Technical Representative to answer questions)

REVIEW OF FORMETANATE DRAFT REPORT AGAINST RAW DATA AND WRITING OF QA DATA AUDIT REPORT

• Thursday, 25 June 2009 (p.m.)	Carol Mitchell
• Friday, 26 June 2009	Writing of Report: Carol T. Mitchell and Brenda T. Culpepper

The primary purpose of the study was to characterize the Formetanate acute time-course and dose–response for cholinesterase inhibition in brain and red blood cells (RBC) in post-natal day (PND) 17 rat pups and the effects of Formetanate on motor activity in PND 17 rat pups.

The review team believes that your Formetanate draft report accurately reflects the raw data.

cc: Dr. David Herr, Acting NTB Branch Chief, TAD
Dr. John Rogers, Acting Division Director, TAD